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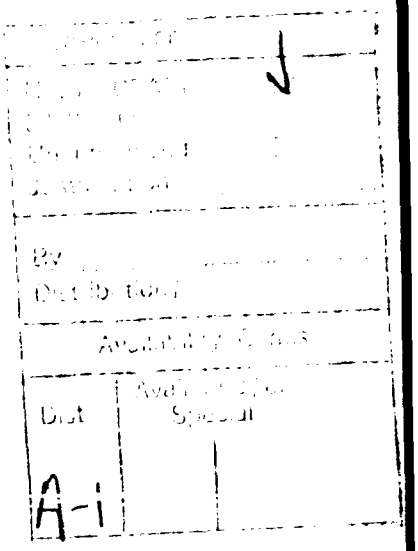

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August 19, 1991

Dear Dr. Haddad

As promised, I am writing to give you a progress report on AFOSR grant 90-0244, "Melatonin, the Pineal Gland and Circadian Rhythms" for 1990. I understand that you need a further report in March of 1992. This project had two interrelated thrusts, both of which are currently in progress. The first, "Cellular mechanisms of melatonin's action using an in vitro hypothalamic slice preparation", has been set up. We have built the apparatus so that we can maintain 16 (from 8 rats) hypothalamic slices simultaneously. Because the in vitro preparation should wash out any anaesthetic, we changed the protocol from the original Newman et al. (J. Neurochem 51 (1988) 1394-1415) protocol to include anaesthesia before sacrifice as was suggested by our local Institutional Animal Care and Use Committee (IACUC). However, our data from this preparation indicated that local glucose utilization (LGU) in the suprachiasmatic nucleus (SCN) in vitro was approximately 1/2 published in vivo LGU's and 1/2 our own preliminary LGU data with unanaesthetized rats! Research by Sokoloff (1984) had already indicated that anaesthesia decreased in vivo LGU by 1/2 as well. This indicates that somehow the effects of Metofane anaesthesia is retained in vitro. This is an interesting effect, and we may return to it later. However, it was not the focus of this project. We then received permission from our IACUC to perform these experiments without anaesthesia. These experiments are currently underway. Our initial data indicate a modest but significant decrease in the relative LGU by 10 nM and 1 μM melatonin but not 10 pM melatonin at CT9-10. However, I am not happy with the control intra-group variability, and we are in the process of improving the preparation.

The second major thrust of this project, "The pineal gland's role in mammalian circadian organization", is progressing very well. We have decided to perform the last experiment listed in the proposal first and have been rewarded with spectacular results, about which I enclose a manuscript which is in press and the J. Biol. Rhythms. It will appear in the first issue of 1992. This study indicates that, although pinealectomy has no effect on rat circadian rhythms in LD or constant darkness (DD), the surgery completely disrupts circadian rhythms in constant light! These effects go away when the rats are returned to DD. These data indicate clearly that, contrary to previous findings, pinealectomy affects rodent circadian rhythms. The effect of pinealectomy, we

hypothesize, is either to increase the photic sensitivity of rats such that they perceive the light intensity to be higher and/or to decrease the amount of coupling among circadian oscillators within the SCN. We will be testing these hypotheses later on this year.

We have also performed experiment 1 from Section 3, "Effects of pinealectomy on entrainment to melatonin injection regimes". This experiment was performed to determine whether 1) the entraining effects of melatonin depended upon the pineal gland and 2) whether the dose-response characteristics of melatonin entrainment were altered by PINX. Male Long-Evans rats (N=72) were maintained in LD 12:12 for 14 days while wheel-running activity was continuously monitored as above. Rats received either a SHAM (N=36) or PINX (N=36) surgery and were allowed to recover for 14 days in LD 12:12. At that time, the LD cycle was delayed 8 hrs and remained there for an additional 14 days. Rats were then placed in DD for 42 days. During this time, rats received subcutaneously injections of 1 of 9 dosages of melatonin ranging from 1 mg/kg to 1 ng/kg body weight at the same time of day every day. They were then allowed to free-run without injections in DD for 14 days. The results indicated that PINX did not block entrainment to exogenous melatonin nor were PINX rats more sensitive to melatonin dosages than were their SHAM controls. The ED_{50} for entrainment was 4.9 $\pm$ 1.5 μg/kg for SHAM rats and 5.2 $\pm$ 2.3 for PINX rats. These values are similar to each other and to previously published values for melatonin entrainment (Cassone et al., 1986). Interestingly, several PINX rats entrained to the melatonin injections aberrantly; some activity components entrained to the regime while others free-ran away. The data indicate that the pineal gland does not contribute to melatonin entrainment *per se* and that the melatonin-sensitive moieties within the brain, presumably within the SCN, are not regulated significantly by endogenous ligand. A manuscript is currently in preparation.

Associated with the research directly involved with the AFOSR project, we have also partially funded a project concerning melatonin receptors using birds. Recent research in this and other laboratories using birds around the world have linked structures subserving circadian function with those involved with visual function. First, application of <u>in vitro</u> binding of IMEL and autoradiography in domestic chick and house sparrow has revealed specific, reversible binding in retinorecipient structures of the circadian (vSCN), tectofugal (TeO) and thalamofugal (LA; DL; GLv) visual systems (Rivkees et al. 1989; Stehle 1990; Cassone and Brooks, in press). In addition, both thalamic relay nuclei (Rt) and telencephalic integrative nuclei (ectostriatum) of the tectofugal visual pathway also bind IMEL in these species. Rivkees et al. (1989) have extensively characterized this binding using whole brain homogenates. These radioreceptor studies revealed a high affinity IMEL binding site with an equilibrium dissociation constant (K_D) of 60 pM and a B_{max} of 23 fmol/mg protein. Binding in this preparation was highly selective for IMEL and melatonin, since competition assays revealed inhibition constants (K_i) significantly lower for IMEL and genuine melatonin than for 6-chloromelatonin, 6-hydroxymelatonin and N-acetylserotonin. Norepinephrine and serotonin, moreover, were ineffective in inhibiting IMEL binding in mM concentrations. In this original study, IMEL reversibility was problematic, since IMEL binding at 50 pM was reduced by only 25% by addition of 1 μM melatonin 1 hr after initial incubation. However, we have repeated this study using <u>in vitro</u> autoradiography and have found an 80% decline using this technique. Because pineal melatonin is synthesized and secreted rhythmically (cf. Takahashi et al. 1989), we were interested in determining the temporal

profile of IMEL binding in the avian brain. White leghorn chicks (Gallus domesticus, N=60) were maintained in brooders for 14 days in LD 12:12 with food (Purina Startena) and water continuously available. At 14 days, 5 birds were removed from the brooder at different times of day and were anesthetized with 80 mg/kg ketamine and 20 mg/kg xylazine (KET/ROM). The times at which birds were anaesthetized were 2 hours after the lights were turned on, or Zeitgeber time (ZT) 2 (0800 CST), then every four hours after at ZT 6 (1200), ZT 10 (1600), ZT 14 (2000), ZT 18 (2400),and ZT 22 (0400). During the dark phase of day 14, the clock-controlled lighting was disabled so that the lights would not turn on the next day. Birds were anaesthetized throughout this following day in complete darkness at the times after lights-on would have occurred: circadian time (CT) 2, CT 6, CT 10, CT 14, CT 18, and CT 22. The birds were transcardially perfused with 50 ml cold 0.75% saline followed by 50 ml cold 0.1 M phosphate buffer (pH= 7.4) containing 10% sucrose. The brains were removed, frozen in isopentane (-40°C), and stored in sealed containers at
-90°C.

Serial coronal sections (20 μm) were cut through the rostrocaudal extent of the brains on a cryostat. The sections were thaw-mounted onto gelatin-coated slides and stored at -20°C. Sections from each brain were divided into experimental groups consisting of adjacent sections in each statistical bin. For the binding assay, the sections were warmed to room temperature and placed into preincubation buffer [0.02 M $NaPO_4$ buffered 0.15 M saline, pH 7.4, containing 0.1% BSA (PBS/BSA); 1 h at 21 C], incubation buffer [one of several concentrations of 2-[^{125}I]iodomelatonin (IMEL, Amersham Corporation, Deerpark, IL) in PBS/BSA, 1 h at 21 C], and wash buffers (PBS/BSA, 15 min at 0°C, followed by PBS alone, 15 min at 0°C). For the binding site characterization experiment, the concentration of IMEL used was from 5 pM to 500 pM. For the binding density experiment, 75 pM IMEL was used. To determine nonspecific binding, alternate sections were placed in incubation buffer containing IMEL plus 1 μM genuine melatonin.

The sections were dried and apposed to ß-Max Hyperfilm (Amersham) for 7 days at -90°C to generate autoradiographs. Radioactive standards (^{125}I microscales, Amersham) were placed on each film to allow quantitative analysis of the binding densities. The binding densities were measured using a computerized image analysis system (Jandel JAVA system). The sections were cleared and stained with cresyl violet to allow anatomical localization of labeled structures. Saturation experiments were analyzed using the non-linear curve fitting computer program, EBDA/LIGAND (Munson 1980).

Scatchard analysis of saturation experiments revealed a high affinity binding site in each structure examined. The K_D and B_{max} of the TeO (K_D= 18.4 pM; B_{max}= 15.3 fmole/mg) was similar to that described by Rivkees et al. (1989) for whole brain and identical to that described by Stehle (1990) for TeO homogenates. Other structures exhibited K_D values which were similar to TeO. However, B_{max} varied tremendously. Kinetic studies with an IMEL concentration of 100 pM indicated that binding reached equilibrium by 110 min at 21°C. Furthermore, 80% of bound IMEL was displaced by the addition of 1 μM melatonin. Analysis of variance indicated a significant peak in binding density around ZT 10 in LD and around CT 10 in DD. There was an increase in amplitude in the rhythm during DD in all structures examined. This interesting fact suggests that light may influence IMEL binding itself. Because the birds in this study

were intact, we were initially concerned that the rhythm we observed was the result of endogenous pineal melatonin remaining in the excised tissue and competing with IMEL. We did not believe this for two reasons: 1) The first is that the phase-relationship of the IMEL binding rhythm to published melatonin rhythmicity did not corroborate this theory: IMEL binding peaks at CT 10 and remains high into the early night when melatonin is high. Conversely IMEL binding is lowest at CT 2 when there is no melatonin in the blood. 2) The second is that previous research (Rivkees et al. 1989) indicated that IMEL had a higher affinity for the putative receptors than did melatonin. Nonetheless, we wanted to test this theory again directly. To determine whether exogenous melatonin pre-incubation could modify binding, sections of daytime tissue (when IMEL binding is high and endogenous melatonin is normally low) were pre-treated with a 1 h incubation of 27 nM melatonin (the melatonin concentration in chick brain tissue at midnight; Cassone et al. 1986) in PBS/BSA, then processed with 50 pM IMEL binding assay. Also, sections of nighttime tissue were placed in preincubation buffer at temperatures from 4-37°C then processed with the 50 pM IMEL binding assay. Because the affinity of putative melatonin receptors to melatonin are known to be temperature-sensitive (Dubocovich 1988), this procedure should have eluted any endogenous melatonin residing in the tissue. Pre-incubation of daytime brain tissue with melatonin (27 nM) did not lower the binding density over controls while increasing incubation temperature from 4-37°C did not increase the IMEL binding density of nighttime tissue. These two preliminary studies corroborate the view that endogenous melatonin is not competing directly with IMEL binding. The data indicate clearly that, in contrast to the apparent case in mammals (see above), avian melatonin receptors are regulated by the circadian system. A manuscript will be submitted to J. Neuroscience this week. We will send you a copy of this manuscript when the figures come back from our Biomedical Productions Dept.

We have begun to ask similar questions in mammals. Although I did not propose the following collaborative experiments, I have taken it on myself to perform them with a combination of AFOSR grant funds and my Texas A&M set-up account. First, in collaboration with Drs. Frank Bronson and Paul Heideman, we have used the in vitro autoradiographic technique for localizing and characterizing melatonin receptors in the brains of two species of mouse, the white-footed mouse, Peromyscus leucopus, and the cane mouse, Zygodontomys brevicauda. The white-footed mice had been bred to be either photoperiodic (and sensitive to melatonin) or not photoperiodic (and not sensitive to melatonin). The cane mice are not photoperiodic under any circumstances. Yet, their melatonin production profiles do exhibit photoperiodic modulation. Mice of both species were maintained in long or short photoperiods (N = 12/photoperiod/species). We have perfused these mice, sectioned their brains and incubated a sequence of sections in 100 pM IMEL and one of 9 concentrations of cold melatonin. This cold displacement technique allowed us to obtain affinity and receptor number data for all structures binding the hormone. The white-footed mouse brains, as was indicated by our previous work in rats, showed no changes in IMEL binding due to photoperiodic history. However, the cane mouse brains were absolutely devoid of IMEL binding, indicating that perhaps this species lack of response is due to its lack of receptor. This work has been presented to the International Society for Chronobiology in Israel by Dr. Bronson in July.

The second collaboration is with Dr. Rae Silver of Columbia University. It is based upon their unusual observation that adult Syrian hamsters do not entrain to

melatonin injections, adult SCN-lesioned hamsters do not entrain, but if SCN-lesioned hamsters are implanted with fetal SCN, the newly rhythmic hamsters do entrain! We are currently collecting a developmental series of hamster fetal and pup brains. We are also collecting brains from hamsters with SCN grafts and will determine if melatonin receptor number or affinity in the grafts correlate with 1) the developmental series and 2) the behavioral effects of the hormone.

A third collaboration has been instigated by Dr. Janet Darrow of Wellesley College in which we will determine the distribution and affinity of melatonin receptors in pinealectomized and sham operated Siberian hamsters whose circadian patterns of activity have been entrained by infusions of melatonin.

I'm sorry for the tardiness of this report. As you know, there was a misunderstanding concerning my previous report to Dr. Berry. I'm very excited about our completed work and even more excited about the work to come!

Sincerely Yours,

Vincent M. Cassone, Ph.D.
Assistant Professor of Biology